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Development of a generator to power ATP-driven molecular motors.

Final Report
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Carlo Montemagno, Ph.D.
Cornell University

EXPERIMENTAL RESULTS

Purification of enzymes.

We were unable to obtain reconstitutionally active TF_1F_0 using the method of Kagawa and Sone (1979), and developed an improved purification protocol for this enzyme that utilizes the detergent n-Dodecyl β-D-Maltoside (Hazard et. al, manuscript in preparation). Bacteriorhodopsin was purified using the method of Dencher and Heyn (1982).

Preparation of proteolipsomes.

Liposomes were prepared by sonicating lipid/cholesterol mixtures in 3:1 diethyl ether: aqueous buffer; removing organic solvent by rotary evaporation; and filtering liposomes successively through 0.45 and 0.22 μm filters (Richard et al., 1995; Rigaud et al., 1983; Szoka and Papahadjopoulos, 1978). The final composition of liposomes was 20 mM lipid (9:1 phosphatidyl choline: phosphatidic acid), 6 mM cholesterol. This composition was chosen as it has been shown to optimize TF_1F_0 ATP synthesis activity (Pitard et al., 1996, Eur J Biochem. 235, 769).

Proteoliposomes were formed by protocol adapted from Sone et al. (1975). Briefly, TF_1F_0, bacteriorhodopsin, and liposomes were solubilized in a solution containing 20 mM cholate, 10 mM deoxycholate. Pyranine (200 μM) was included as desired for measurement of internal pH. Samples were diluted fourfold with Dialysis Buffer (20 mM MOPS, 50 mM Na_2SO_4, 50 mM K_2SO_4, 2.5 mM $MgSO_4$, 0.25 mM DTT, 0.2 mM EDTA), and dialyzed overnight at 4°C against three changes of the same buffer. Samples stored at 4°C, and retained activity for up to two weeks.

Measurement of pH gradient formation.

Change in internal pH was determined using pyranine fluorescence as previously described (Overly et al., 1995; Pitard et al., 1996). Light-dependent pH gradient formation by bacteriorhodopsin was activated using an Intralux xenon lamp fitted with a yellow filter, and ATP-dependent pH gradient formation by TF_1F_0 was initiated by the addition of 15 mM MgATP. **Figure 1** illustrates typical data for bacteriorhodopsin (*closed circles*) and TF_1F_0 (*open circles*). Bacteriorhodopsin typically produced a maximum pH gradient of ~1 unit, while TF_1F_0 produced a pH gradient of ~0.5 units. For bacteriorhodopsin- TF_1F_0 proteoliposomes, a light-driven pH gradient was maintained for at least 30 minutes dark incubation, indicating that proteoliposomes are adequately proton-impermeable. Both light- and ATP-dependent pH gradient formation were reversed by the addition of the uncoupler CCCP, and ATP-dependent pH gradient formation was inhibited by the F_0-inhibitor DCCD (data not shown).

DISCLAIMER

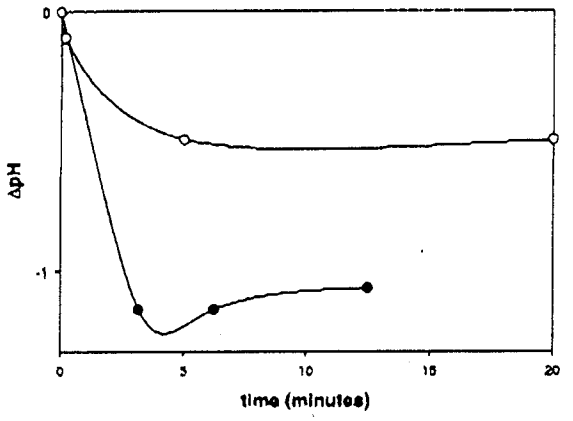

Figure 1. *Closed circles*- Light-driven pH gradient formation by bacteriorhodopsin; sample contained 20 μg/ml TF_1F_0, 138 μg/ml bacteriorhodopsin, 3.3 mM liposome. A maximum ΔpH of 1.1 unit was reached within 3 minutes. *Open circles*- ATP-driven pH gradient formation by TF_1F_0. Sample contained 0.02 μg/ml TF_1F_0 and 3.3 mM lipsome. To ensure that no light-driven proton pumping could occur, no bacteriorhodopsin was present. A maximum ΔpH of 0.7 units was reached within 5 minutes.

Measurement of ATP synthesis activity.

Bacteriorhodopsin and TF_1F_0 were reconstituted into proteoliposomes as described above. Samples were illuminated for five minutes to obtain a maximum pH gradient, at which point the ATP synthesis reaction was initiated by the addition of 4 mM Mg-ADP, 20 mM Pi. Aliquots were removed at the indicated times, and the reaction stopped by the addition of 2 % TCA. ATP was quantified using the luciferin/luciferase assay kit from Sigma (Sigma #FL-AA). A typical ATP synthesis reaction is illustrated in **Figure 2**. Curiously, the ATP synthesis rate increases with time up to sixty minutes. This rate increase is does not appear to be related to an increase in the pH gradient, as ATP synthesis was not initiated until the pH gradient was at a maximum. To test the possibility that substrate binding causes a slow activation of the enzyme, samples were preincubated with Mg-ADP or with Pi for 30 minutes prior to initiation of the ATP synthesis reaction; the results obtained were identical whether or not proteoliposomes were preincubated with either substrate. Richard et al. observed a similar increase in ATP synthesis rate for TF_1F_0, which was unaffected by the duration of light-preincubation, or by preincubation with substrates (1995). These results suggest that TF_1F_0 is slowly activated by enzymatic turnover. By contrast, the same authors observed that the chloroplast F_1F_0 ATP synthesis rate remained unchanged over time.

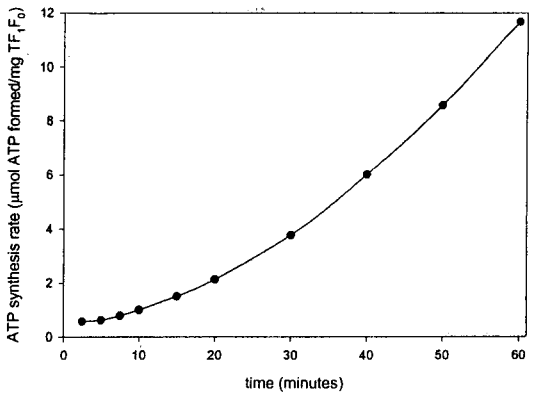

Figure 2. *Typical ATP synthesis reaction.* Sample contained 30 µg/ml TF_1F_0, 413 µg/ml bacteriorhodopsin, 5 mM liposome. Experiments were done at 30°C. Similar results were obtained for experiments done at 25 and 42°C (data not shown). Data represent an average of three measurements.

As the ATP synthesis rate changes with time, it is necessary to indicate at what time point activity was measured. At sixty minutes, the sample shown in Figure 2 had an activity of 193 nmol/min/mg. The composition of proteoliposomes in this sample was 30 µg/ml TF_1F_0, 413 µg/ml bacteriorhodopsin, 5 mM lipid. Similar activities were obtained when the bacteriorhodopsin concentration was varied between 138 and 600 µg/ml at a set TF_1F_0 concentration, or when the TF_1F_0 concentration was varied between 20-60 µg/ml at a set bacteriorodopsin concentration. Furthermore, the magnitude of the pH gradient was unaffected by the initiation of ATP synthesis. These results indicate that, within the TF_1F_0 concentration range tested, ATP synthesis is not being limited by bacteriorhodopsin concentration. Thus, the observed ATP synthesis rate presumably represents the maximum achievable rate for TF_1F_0 under the experimental conditions tested.

SUMMARY

Here, we report a maximum ATP synthesis rate of 193 nmol/min/mg for thermophilic F_1F_0. This rate is somewhat lower than the previously observed maximum rate of 500-700 nmol/min/mg (Pitard et al., 1996). However, ATP synthesis rates vary considerably with experimental conditions, and our observed rates compare favorably with the wide range of rates (40-700 nmol/min/mg) observed by these authors. Future research will focus on maximizing the ATP synthesis rate by adjusting environmental conditions, including the lipid and cholesterol composition of the proteoliposomes.

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